Establishment and Characterization of Benzo(a)pyrene-Induced Skin Tumor in Rats

(PEMBERUNTUKAN DAN KARAKTERISASI TUMOR KULIT PADA TIKUS YANG DIPICU OLEH BENZO(A)PIREN)

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ABSTRACT

Quite a number of research on cancer therapy strongly require an animal model of cancer. One of the chemicals commonly used to induce cancer in animal models is benzo(a)pyrene due to its carcinogenic effects. This study aims were to describe the gross pathology of the tumor-induced by benzo(a)pyrene in an olive oil solution (w/v), identify the type of tumor histopathologically, and finally, determine the correlation between the duration of the rats experiencing tumor and it’s grade score. Tumor grade score is important to assess in order to determine tumor malignancy. This study consisted of 10 white rats (Rattus norvegicus) were given two treatments: a negative control treatment (K-) was injected with 0.1 mL of olive oil and a positive control treatment (K+) was injected with 0.1 mL of 0.3 % (w/v) benzo(a)pyrene in olive oil solution. Each treatment rats was kept in a cage and monitored regularly. When the tumors macroscopically appeared in the interscapular area and were observed until reached 4 cm in size, the rats were then sacrificed and necropsied. Tumors were observed for the gross pathology to examine the shape and color of them, then routinely processed for histopathological evaluation. The results showed that the tumors’ cells appeared to be round (1/5), irregular (2/5), and multilobular (2/5). Based on histopathological observation, the types of tumors observed were classical fibrosarcoma (2/5) and pleomorphic fibrosarcoma (3/5). There is a significant association between the duration of the rats experiencing tumors and the tumor grade. The longer the rats have tumors, the tumors tend to be more aggressive.

Keywords: benzo(a)pyrene; skin tumor; subcutaneous; rats; malignant tumors

ABSTRAK

Banyak penelitian mengenai terapi kanker membutuhkan hewan model penderita kanker. Salah satu bahan kimia yang sering digunakan untuk menginduksi kanker pada hewan coba adalah benzo(a)piren yang bersifat karsinogenik. Studi ini bertujuan untuk mendeskripsikan lesi patologi anatomi dari tumor yang dipicu oleh benzo(a)piren dalam larutan minyak zaitun (w/v), mengidentifikasi jenis tumor secara histopatologis dan menentukan korelasi antara durasi tikus yang mengalami tumor dengan skor grade tumor. Skor grade tumor sangat penting untuk mengukur tingkat keganasan tumor. Studi ini menggunakan 10 ekor tikus putih (Rattus norvegicus) yang diberikan dua perlakuan: kontrol negatif (K-) diinjeksi dengan 0,1 mL minyak zaitun sedangkan kontrol positif (K+) diinjeksi dengan 0,1 mL larutan yang mengandung...
INTRODUCTION

Cancer or malignant tumor is a mass in tissue that arises from uncontrolled growth due to genetic mutations. The growth of malignant tumors occurs continuously and completely ignores normal cell regulatory processes (Gamoudi and Blundell, 2008). Genetic mutations can be caused by exposure to radiation, hormonal factors, chemical substances, chronic irritants, parasites, and oncogenic viruses (Blackadar, 2016). Globally, this disease has been declared to be the second killer for humans (WHO, 2021).

The incidence of malignant tumors is influenced by the immune response, nutritional requirements, and the vascularity of transformed cells (Scott and Wille, 1984; Nerlich et al., 2006; Saman et al., 2020). Research in cancer is importantly needed like some other gaps of it such as to better understand the biology of cancer, evaluate tumor progression in the preclinical study, and develop new therapies. Therefore, experimental animals are needed to be the models for the cancer patient. Rats are one of the experimental animals that are often used to be the model. There are several techniques established in animal models with cancer, including spontaneous tumor models, cancer-induced animal models with oncogenic viruses, induction with radiation, chemical induction, and xenograft models (Navale, 2013).

A common preferable method for inducing cancer in an animal model is using chemical induction with benzo(a)pyrene. Benzo(a)pyrene is often used because it is easier being obtained, and its application is easy compared to other techniques. Benzo alpha pyrene or benzo(a)pyrene is a member of the Polycyclic Aromatic Hydrocarbons (PAHs). These substances are commonly known as environmental pollutants and their metabolites are carcinogenic. In the environment, these compounds can be found in air, food, and water (Agarwal et al., 2018).

Benzo(a)pyrene is the strongest carcinogenic agent among other PAH members, so it is often used for the induction of malignant tumors in animal models for cancer study purposes (Fitzgerald et al., 2004; Gu et al., 2013; Gu et al., 2018). The metabolite is benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide, which is highly toxic and carcinogenic. It can go through the chemical carcinogenesis stage, i.e. initiation stage (Schwarz et al., 2001).

Many in vivo studies have used benzo(a)pyrene as an inducer agent for malignant tumors, one of them is the study conducted by Kallistratos and Fasske (1980) that injecting benzo(a)pyrene dissolved in tricaprylin via the subcutaneous route in Wistar rats. The combination of benzo(a)pyrene and tricaprylin causes several types of tumor cells, including fibrosarcoma, rhabdomyosarcoma, and polymorph cell sarcoma. Other studies that used benzo(a)pyrene for the induction of malignant tumors, specifically to get fibrosarcoma in rats have been carried out by combining benzo(a)pyrene with olive oil (Sukardiman et al., 2015; Sewoyo et al., 2021a; Sewoyo et al., 2021b).

Various types of malignant tumors can arise from the skin, which classifies them into six main groups, including keratinocytic tumors, melanocytic tumors, appendageal tumors, soft tissue tumors, neural tumors, and subcutaneous tissue tumors (Khanpur and Ramam, 2012). Although many studies have conducted subcutaneous cancer induced by benzo(a)pyrene, most of them use many different solvents, which induce non-uniform tumor types. However, the description of gross lesions and histopathological assessment of the tumor are lacking.

In this study, we established a malignant tumor in rats by injecting benzo(a)pyrene solution in olive oil. This study aims were to describe the gross pathology and determine tumor types, as well as evaluate the relationship between the duration of rats with tumors and tumor grade score.
RESEARCH METHODS

Experimental Animals

The experimental animal used in this study was 10 male white rats (Rattus norvegicus) Sprague-Dawley (SD) strain, aged about three months old. Rats were kept in one cage for each treatment. All procedures carried out in this study have been previously approved by the Ethical Committee for the Use of Experimental Animals, Faculty of Veterinary Medicine, Udayana University.

Tumor Induction

After adapting for one week, the rats were randomly divided into two treatment groups. Each treatment group consisted of five rats. All rats in the negative control treatment (K-) was injected with 0.1 mL technical grade olive oil (Oleum olivarum) while those in the positive control treatment (K+) was injected with 0.1 mL of benzo(a)pyrene (Sigma-Aldrich, St. Louis, MO, USA) solution in technical grade olive oil at a concentration of 0.3% (w/v).

Treatment injection of olive oil or benzo(a)pyrene solution for K- and K+ respectively were carried out within 10 times repetition, one injection was given every two days. The injection was performed subcutaneously at the interscapular area of the rats (Sewoyo et al., 2021a; Sewoyo et al., 2021b; Sukardiman et al., 2015). Then, the rats were reared in the same environment and regularly monitored after the first injection. All of the rats were treated humanely, the same feed and drink were given to both groups ad libitum. Referring to the maximum limit of subcutaneous tumor size in rats that allowed for the use of an animal tumor model is 40 mm/4 cm (IAUC, 2019), so when the tumor was reached 4 cm in diameters the rats then were sacrificed.

Gross Examination and Sample Collection

The sampled rats were necropsied and the tumor mass was observed macroscopically before then the tumor sample was taken. The tumors tissues were then fixed in 10% neutral phosphate-buffered formalin for 24 hours at room temperature, embedded in paraffin, cut into 3-µm sections, and stained with hematoxylin-eosin (H&E).

Histopathological Examination

The slide was then observed using a microscope (Olympus BX53, Olympus Corp., Tokyo, Japan) for histopathological observations. Histopathologic determination of the tumor type is based on Greaves et al. (2013). The tumor malignancy was assessed by the most widely recommended grading system as described by Augsburger et al. (2017). Tumor grade scoring/assessment is important for tumor malignancy determination by determining its degree of differentiation, mitotic index, and amount of necrosis (Table 1).

Data Analysis

The shape and types of tumors was presented in tabular form, gross lesion, while the histopathological examination were presented

<table>
<thead>
<tr>
<th>Score A: Tumor differentiation score</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcomas closely resembling normal adult mesenchymal tissue</td>
<td>Sarcoma for which histological typing is certain</td>
<td>Embryonal undifferentiated sarcoma</td>
<td></td>
</tr>
<tr>
<td>Score B: Mitotic activity score</td>
<td>0-9 mitoses per 10 HPF*</td>
<td>10-19 mitoses per 10 HPF</td>
<td>≥20 mitoses per 10 HPF</td>
</tr>
<tr>
<td>Score C: Tumor necrosis score</td>
<td>No necrosis</td>
<td>&lt;50% necrosis</td>
<td>&gt;50% necrosis</td>
</tr>
<tr>
<td>Grade 1: Low grade</td>
<td>Score A+Score B+Score C = 2 or 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 2: Intermediate grade</td>
<td>Score A+Score B+Score C = 4 or 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 3: High Grade</td>
<td>Score A + Score B + Score C = 6, 7, or 8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*HPF (high power field): 1 HPF = 0.1734 mm²
The duration of the rats experiencing tumors and each tumor grade score was then tabulated. The correlation and regression analysis were performed using SPSS Version 25 software for Windows.

RESULTS AND DISCUSSION

Gross Pathology

Negative control rats (K-) that were injected with olive oil showed no signs of neoplastic growth. The interscapular area of the rats was observed normal and no sign of neoplastic growth was found when it palpated. However, those rats treated with benzo(a)pyrene or positive control rats (K+), showed a tumor in their interscapular area (Figure 1). The timing of the tumor visible on the interscapular area post benzo(a)pyrene injection,

Table 2. Gross pathological tumor observation

<table>
<thead>
<tr>
<th>Shape</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Round</td>
<td>(1/5)</td>
</tr>
<tr>
<td>Irregular</td>
<td>(2/5)</td>
</tr>
<tr>
<td>Multilobular</td>
<td>(2/5)</td>
</tr>
</tbody>
</table>

Figure 1. Macroscopic appearance of tumors in rats on the 139th day after injection with benzo(a)pyrene (a) Tumor observed multilobular-shaped, with two lobes on the interscapular area (b) A single lobe tumor on interscapular.

Figure 2. Gross pathology of the tumor after excision (a) there are several variations in the shape of the tumor, i.e. round, irregular, and multilobular. (b) Contents of the tumor after splitted into two pieces. The appearance varies, some are only white and appear solid, some are visible with reddish areas and yellowish-brown areas.
were varied considerably (Table 2). After the tumor appeared, it was then observed until it reached a maximum size of 4 cm. On the 140th day after benzo(a)pyrene injection, the rats were sacrificed and the tumor tissue was excised.

The outer color of the tumor also varies, some areas had slightly pale white, reddish color, and some other areas were yellowish-brown. In order to see the contents of the tumor, it was divided into two parts and found that the tumor was mostly very dense, which might contribute to firm consistency of them, except the tumor no. 2 which was softer and more elastic.

When the tumors were cut into two parts, tumors no. 1 and 4 appeared to have similarities in the cut surface and their color. The cut surface of them were solid and flat, with grayish-white color, while tumor no. 2 had a reddish and a white-gray areas. Tumors no. 3 and 5 were similar in surface and color, the surface was uneven with a yellowish-brown and a dark gray colors.

**Histopathologic Appearance of Rat Tumors**

Tumor type, duration of the rats experiencing tumor, and tumor grade score are presented in Table 3. Tumors no. 1, 2, 3, and 5 seemed to be a massive proliferation of fibroblast cells, which were composed of collagen connective tissue and had a high mitotic index. The fibroblasts in tumor no. 1 had a basophilic nucleus with an almost uniform shape, *i.e.* spindle-shaped cells. The cell shapes varied slightly and no giant cells, bizarre cells, or multinucleated cells found (Figure 3a).

The fibroblasts cells of tumor no. 4 tended to be hyperchromatic with slight cellular atypia. Similar to tumor no. 1, tumor no. 4 did not have giant cells, bizarre cells, or multinucleated cells. Fibroblast cells in tumors no. 2, 3, and 5 had cellular atypia, which means that there are variations in the shape of the fibroblasts. Some fibroblasts appeared to be essentially hyperchromatic (Figs. 3b, c, e).

Multinucleated cells were found in tumors no. 2, 3, and 5, giant cells in tumors no. 3 and 5, and bizarre cells in tumor no. 5. The pattern of fibroblast cell arrangement in tumors no. 2 and 3 is arranged in storiform, while tumor no. 1 is herringbone. Based on these findings, histomorphologic diagnosis of tumor no. 1 and 4 are classical fibrosarcoma, while tumor no. 2, 3, and 5 are pleomorphic fibrosarcoma.

In negative control rats (K-) that injected with olive oil did not show any neoplastic or metaplastic changes in the histological structure of their skin. The structure was still intact, like normal skin histology in general. Olive oil injection did not cause pathologic changes in the samples because it is not carcinogenic.

Based on the correlation and regression analysis there was a significant relationship (*p*-value = 0.019, *p*<0.05) between the duration of the rats experiencing tumors and the malignancy score. It can be concluded that the longer the rats had a malignant tumor, the higher the tumor grade score.

<table>
<thead>
<tr>
<th>Rat number</th>
<th>Day post injection (day)</th>
<th>Duration of having a tumor (day)</th>
<th>Tumor type</th>
<th>Tumor grade score</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>120</td>
<td>20</td>
<td>Classical fibrosarcoma</td>
<td>4</td>
<td>Intermediate Grade</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>50</td>
<td>Pleomorphic fibrosarcoma</td>
<td>8</td>
<td>High Grade</td>
</tr>
<tr>
<td>3</td>
<td>115</td>
<td>23</td>
<td>Pleomorphic fibrosarcoma</td>
<td>5</td>
<td>Intermediate Grade</td>
</tr>
<tr>
<td>4</td>
<td>116</td>
<td>24</td>
<td>Classical fibrosarcoma</td>
<td>4</td>
<td>Intermediate Grade</td>
</tr>
<tr>
<td>5</td>
<td>101</td>
<td>39</td>
<td>Pleomorphic fibrosarcoma</td>
<td>7</td>
<td>High Grade</td>
</tr>
</tbody>
</table>
Figure 3. Histopathologic features of rat tumor induced by a solution of benzo(a)pyrene in olive oil.
(a) Tumor no. 1, classical fibrosarcoma with spindle-shaped cell, basophilic nucleus (black arrow), and cells undergoing mitosis (orange arrow). (b) Tumor no. 2, pleomorphic fibrosarcoma with storiform pattern (asterisk) and multinucleated cells (black arrow). (c) Tumor no. 3, pleomorphic fibrosarcoma with storiform pattern (asterisk), histiocytes (blue arrow), giant cell (black arrow), and cells undergoing mitosis (orange arrow). (d) Tumor no. 4, classical fibrosarcoma with the presence of hyperchromatic spindle-shaped fibroblast cells and slightly cellular atypia, interwoven in a collagenous tissue (black arrow). (e) Tumor no. 5, pleomorphic fibrosarcoma with the presence of bizarre cells (black arrow), giant cells (orange arrow), and cells undergoing mitosis (blue arrow). (f) Histological structure of rat skin injected with olive oil. There is no sign of neoplastic changes. Note: Scale bar figure a,b,c,d,e = 200 µm, figure f and insert figure a,c,d,e bar 50 µM. H&E Staining
Discussion

The rat fibrosarcoma models in this study were established by injecting a solution of benzo(a)pyrene in olive oil. The tumors that macroscopically appeared had distinctive features. Microscopically, classical fibrosarcoma is characterized by the presence of massive fibroblast cell proliferation with a uniform or monomorphic shape, slightly cellular atypia, and no giant cells or multinucleated cells. The typical arrangement of classical fibrosarcoma is that the tumor cells are generally arranged in a herring bone pattern, while pleomorphic fibrosarcomas are found several cellular atypia, multinucleated cells, and giant cells arranged in a storiform pattern (Greaves et al., 2013).

Tumor no. 2 tended to be softer compared to the others (tumor no. 1, 3, 4 and 5). This is likely due to fibroblasts in tumor no. 2 were more dominant than collagen connective tissue. The other tumors might have more collagen tissues, which made their consistency harder than the tumor no. 2.

Malignant tumors have a fast growth rate, infiltrative, invasive to the surrounding tissue, and are usually multilobular-shaped. Likewise, benign tumors have a slow growth rate and do not invade nearby tissue (Baba and Câtoi, 2007; Chauhan, 2010).

As described previously, benzo(a)pyrene will turn into a carcinogen through bioactivation with cytochrome P450 CYP1A1 and/or CYP1B1 enzymes. The bioactivation product of benzo(a)pyrene is benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide metabolite (Schwarz et al., 2001). These metabolites will react with DNA, then irreversible damage will occur. This phenomenon reflects the initiation stage in the chemical carcinogenesis process.

To induce malignant tumors, mutations are needed in several genes encoding growth or normal cell cycle regulators, because there is no single genetic defect that can cause malignant tumors (Knudson, 2001). Common genes that have mutations are proto-oncogenes (activation into oncogenes, which triggers a proliferation signal) or mutations in the tumor protein p53/TP53 (inactivation occurs, so it cannot suppress the abnormal proliferation signal) (Rundhaug and Fischer, 2010; Rivlin et al., 2011; Muir and Nunney, 2015).

Nearly more than 50% of the incidence of malignant tumors have mutations in the TP53 gene (Sobhani et al., 2020). In humans, fibrosarcomas arise from defects in the following genes: TP53, cyclin-dependent kinase inhibitor 2A (CDKN2A), CDKN2B, Ewing sarcoma breakpoint region 1 (EWSR1), and alpha-thalassemia X-linked (ATRX) (AACR Project GENIE Consortium, 2017). Fibrosarcomas arise from the subcutaneous, or other areas that have a lot of collagen tissue (Augsburger et al., 2017).

The role of olive oil is to slow the diffusion of benzo(a)pyrene, so that it may last longer in the subcutaneous tissue. The slow diffusion is due to the poor solubility of olive oil (Reddy and Fialkow, 1981). The use of solvents that have a high level of solubility allows many types of tumor cells to appear (Reddy and Fialkow, 1981; Slaga and Fischer, 1981). A Study by Reddy and Fialkow (1981) has shown that methylcholanthrene dissolved in a high solubility solvent benzene, which can induce rhabdomyosarcoma and fibrosarcoma. When methylcholanthrene dissolved in olive oil, it only induced fibrosarcoma. The emergence of rhabdomyosarcoma occurs because the carcinogenic compounds that dissolved by high solubility benzene, which can diffuse rapidly. As a result, carcinogen compounds can reach the muscular tissue and cause rhabdomyosarcoma (Reddy and Fialkow, 1981). It can be concluded that the nature of the solvent plays an important role in tumor type.

In studies using chemical agents as cancer inductors, especially with benzo(a)pyrene, it has several limitations, one of them is that it takes a long time to induce cancer (Cekanova and Rathore, 2014; Sewoyo et al., 2021a). In addition, the onset of cancer tends to be non-uniform (Sewoyo et al., 2021a). However, the combination of benzo(a)pyrene and olive oil has the advantage that the type of tumor that forms tends to be uniform, i.e. fibrosarcoma. Based on the results of the study, the longer the tumor, the higher the tumor grade score. A low tumor score indicates the tumor is less aggressive, and the prognosis tends to be better. Conversely, the higher the scores indicate the tumor characteristics are more aggressive and may lead to poor prognosis (Augsburger et al., 2017).

CONCLUSION

Subcutaneous injection of benzo(a)pyrene in olive oil into rats can cause tumors with various shapes i.e. round tumors (1/5), irregular (2/5), and multilobular (2/5). From the results of histopathological observations, it can be
concluded that the combination of benzo(a)pyrene and olive oil can produce uniform tumors, i.e. fibrosarcoma, with two types, namely classical fibrosarcoma (2/5) and pleomorphic fibrosarcoma (3/5). There is a significant association between the duration of the rats experiencing tumors and the tumor grade score. The longer the rat has the tumor, the more aggressive the tumors.

**SUGGESTION**

The preparation of animal fibrosarcoma models by inducing with benzo(a)pyrene in addition to having advantages also has weaknesses. One of the drawbacks is that if the tumor is left too long, its malignancy tends to be non-uniform and it leads to a poor prognosis, so trials should use tumors that are not too large. Suggestions for research that requires data on tumor volume, should be done in the early stages of tumor formation because in the chronic phase it tends to be multilobular, making it difficult to measure the volume.

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